



TITLE:

Role of PGE-type receptor 4 in auditory function and noise-induced hearing loss in mice.

AUTHOR(S):

Hamaguchi, Kiyomi; Yamamoto, Norio; Nakagawa, Takayuki; Furuyashiki, Tomoyuki; Narumiya, Shuh; Ito, Juichi

CITATION:

Hamaguchi, Kiyomi ...[et al]. Role of PGE-type receptor 4 in auditory function and noise-induced hearing loss in mice.. *Neuropharmacology* 2012, 62(4): 1841-1847

ISSUE DATE:

2012-03

URL:

<http://hdl.handle.net/2433/153283>

RIGHT:

© 2011 Elsevier Ltd.; This is not the published version. Please cite only the published version.; この論文は出版社版ではありません。引用の際には出版社版をご確認ご利用ください。

Role of PGE-type receptor 4 in auditory function and noise-induced hearing loss in mice

Kiyomi Hamaguchi¹, Norio Yamamoto¹, Takayuki Nakagawa¹, Tomoyuki Furuyashiki^{2, 3}, Shuh Narumiya^{2, 3}, Juichi Ito¹

¹Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan

²Department of Pharmacology and ³Japan Science and Technology Agency, Core Research for Evolutional Science and Technology (CREST), Kyoto University Faculty of Medicine, Kyoto 606-8501, Japan

Corresponding author:

Takayuki Nakagawa

Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kawaharacho 54, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

Phone: 81-75-751-3346, Fax: 81-75-751-7225

E-mail: tnakagawa@ent.kuhp.kyoto-u.ac.jp

Abstract

This study explored the physiological roles of PGE-type receptor 4 (EP4) in auditory function. EP4-deficient mice exhibited slight hearing loss and a reduction of distortion-product otoacoustic emissions (DPOAEs) with loss of outer hair cells (OHCs) in cochleae. After exposure to intense noise, these mice showed significantly larger threshold shifts of auditory brain-stem responses (ABRs) and greater reductions of DPOAEs than wild-type mice. A significant increase of OHC loss was confirmed morphologically in the cochleae of EP4-deficient mice. Pharmacological inhibition of EP4 had a similar effect to genetic deletion, causing loss of both hearing and OHCs in C57BL/6 mice, indicating a critical role for EP4 signaling in the maintenance of auditory function. Pharmacological activation of EP4 significantly protected OHCs against noise trauma, and attenuated noise-induced hearing loss in C57BL/6 mice. These findings suggest that EP4 signaling is necessary for the maintenance of cochlear physiological function and for cochlear protection against noise-induced damage, in particular OHCs. EP4 might therefore be an effective target for cochlear disease therapeutics.

Key words: cochlea; hair cell; hearing loss; noise trauma; PGE-type receptor.

Role of PGE-type receptor 4 in auditory function and noise-induced hearing loss in mice

Kiyomi Hamaguchi¹, Norio Yamamoto¹, Takayuki Nakagawa¹, Tomoyuki Furuyashiki^{2, 3}, Shuh Narumiya^{2, 3}, Juichi Ito¹

¹Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan

²Department of Pharmacology and ³Japan Science and Technology Agency, Core Research for Evolutional Science and Technology (CREST), Kyoto University Faculty of Medicine, Kyoto 606-8501, Japan

Corresponding author:

Takayuki Nakagawa

Department of Otolaryngology, Head and Neck Surgery, Graduate School of Medicine, Kyoto University, Kawaharacho 54, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

Phone: 81-75-751-3346, Fax: 81-75-751-7225

E-mail: tnakagawa@ent.kuhp.kyoto-u.ac.jp

Abstract

This study explored the physiological roles of PGE-type receptor 4 (EP4) in auditory function. EP4-deficient mice exhibited slight hearing loss and a reduction of distortion-product otoacoustic emissions (DPOAEs) with loss of outer hair cells (OHCs) in cochleae. After exposure to intense noise, these mice showed significantly larger threshold shifts of auditory brain-stem responses (ABRs) and greater reductions of DPOAEs than wild-type mice. A significant increase of OHC loss was confirmed morphologically in the cochleae of EP4-deficient mice. Pharmacological inhibition of EP4 had a similar effect to genetic deletion, causing loss of both hearing and OHCs in C57BL/6 mice, indicating a critical role for EP4 signaling in the maintenance of auditory function. Pharmacological activation of EP4 significantly protected OHCs against noise trauma, and attenuated noise-induced hearing loss in C57BL/6 mice. These findings suggest that EP4 signaling is necessary for the maintenance of cochlear physiological function and for cochlear protection against noise-induced damage, in particular OHCs. EP4 might therefore be an effective target for cochlear disease therapeutics.

Key words: cochlea; hair cell; hearing loss; noise trauma; PGE-type receptor.

Abbreviations

4',6-diamino-2-phenyl-indole (DAPI)

Auditory brainstem response (ABR)

E-prostanoid receptors (EP)

Prostaglandin E₂ (PGE₂)

Prostaglandins (PGs)

analysis of variance (ANOVA)

dimethyl sulfoxide (DMSO)

distortion-product otoacoustic emissions (DPOAEs)

hematoxylin and eosin (H&E)

inner hair cells (IHCs)

noise floor (NF)

outer hair cells (OHCs)

round window membrane (RWM)

sound pressure level (SPL)

1. Introduction

Prostaglandins (PGs) are short-lived potent bioactive lipid messengers belonging to the family of eicosanoids, and are involved in numerous physiological reactions (Funk, 2001; Harris et al., 2002; Simmons et al., 2004; Smith et al., 2000). Prostaglandin E₂ (PGE₂) is the most abundant prostanoid in humans, and is involved in the regulation of various fundamental biological functions including hemodynamics and inflammation (Legler et al., 2010; Narumiya, 2007). The divergent effects of PGE₂ signaling probably depend on the distinct patterns and dynamics of the expression of PGE receptors, particularly E-prostanoid receptors 1–4 (EP1–4) (Sugimoto and Narumiya, 2007). For example, in hemodynamics, EP1 induces vasoconstriction in the peripheral vasculature, whereas EP2 and EP4 induce vasodilation.

There is growing evidence that individual EP signaling plays significant roles in neurodegenerative diseases (Andreasson, 2010). Although some subtype-specific EP signaling events mediate toxic effects in the central nervous system, others paradoxically appear to mediate protective effects (Andreasson, 2010). EP4 signaling has been reported to provide neuroprotective effects in excitotoxic (Ahmad et al., 2005) and ischemic (Li et al., 2008) models, and, in some contexts, to mediate an anti-inflammatory effect (Esaki et al., 2010; Shi et al., 2010). Pharmacological

inhibition of EP4 signaling reverses the attenuation of spinal cord injury caused by PGE₁ analogs (Umemura et al., 2010). These findings in the central nervous system encouraged us to investigate the roles of EP4 signaling in the auditory system.

In the inner ear, PGs have attracted particular attention as regulators of the cochlear blood flow (Rhee et al., 1999; Tominaga et al., 2006; Umemura et al., 1990), and disorders of this blood flow have been considered a principle cause of sudden deafness (Nakashima et al., 2003). This has resulted in the clinical use of PGE₁ in the treatment of sudden deafness, although little is known about the roles of PGE₂–EP signaling in auditory function (Nakagawa, 2011). Previous studies have demonstrated that PGE₂ is constitutively produced in the cochlea (Kawata et al., 1988) and that all EP subtypes are expressed in various types of cochlear cell (Hori et al., 2009, 2010; Stjernschantz et al., 2004), suggesting that EP signaling might play roles in the physiology and pathophysiology of the cochlea. The present study focused on EP4 because of its reported protective effects on neurons against neuronal degeneration in various contexts (Ahmad et al., 2005; Esaki et al., 2010; Li et al., 2008; Shi et al., 2010; Umemura et al., 2010).

In the current study, we evaluated the functionality and morphology of cochleae in EP4-deficient mice under physiological conditions or following noise

exposure. Pharmacological inhibition or activation of EP4 signaling was examined using mouse models of noise-induced hearing loss in order to confirm the findings.

2. Materials and methods

2.1. *Animals and reagents*

EP4-deficient mice were generated as described previously (Segi et al., 1998). Most EP4-deficient mice die postnatally as a result of patent ductus arteriosus and do not survive on a C57BL/6 background (Nguyen et al., 1997; Segi et al., 1998). Male EP4-deficient mice with a mixed 129/Ola and C57BL/6 genetic background (Kabashima et al., 2002) aged 8 weeks were used. Male mice with a similar mixed genetic background to the EP4-deficient mice were used as wild-type animals. The pharmacological inhibition and enhancement of EP4 signaling were investigated using 8-week-old male C57BL/6 mice (Japan SLC, Inc., Hamamatsu, Japan).

Animals were maintained at the Institute of Laboratory Animals, Kyoto University, Japan, under a 12-h light/12-h dark cycle and specific pathogen-free conditions. Mice were fed *ad libitum* with standard chow. Bedding and water bottles were replaced daily. The Animal Research Committee of the Graduate School of Medicine, Kyoto University, approved all experimental protocols.

The EP4 antagonist ONO-AE3-208 and the EP4 agonist ONO-AE1-329 were supplied by Ono Pharmaceutical, Co., Ltd (Osaka, Japan). Rabbit anti- β III tubulin antibody was purchased from Covance Research Products (Berkeley, CA). Rabbit

anti-myosin VIIa polyclonal antibody was purchased from Proteus BioSciences (Ramona, CA). Alexa 488 or 568-conjugated goat anti-rabbit antibody, DAPI, and fluorescein-phalloidin were purchased from Molecular Probes (Eugene, OR). Rabbit anti-EP4 receptor polyclonal antibody was purchased from Caymann Chemical (Ann Arbor, MI).

2.2. *Auditory brainstem response (ABR)*

Measurements of ABRs have been performed as described previously (Kada et al., 2009). Thresholds were determined for frequencies of 10, 20, and 40 kHz from a set of responses at varying intensities with 5-dB sound pressure level (SPL) intervals. When no response was present at the highest sound level available, the threshold was designated as being 5 dB greater than that level for statistical purposes. The thresholds at each frequency were verified at least twice.

2.3. *Distortion-product otoacoustic emissions (DPOAEs)*

Recordings were made with an acoustic probe (ER-10C; Etymotic Research, Elk Grove Village, IL) using the DP2000 DPOAE measurement system version 3.0 (Starkey Laboratory, Eden Prairie, MN). Two primary tones with an f_2/f_1 ratio of 1.2 were

presented at intensity levels of 65 dB SPL (L1) and 55 dB SPL (L2). The f_2 was varied in one-ninth-octave steps from 8 to 16 kHz. A peak at $2f_1-f_2$ in the spectrum was recognized as a DPOAE. The DP/ noise floor (NF) levels were calculated and statistical analyses were performed at each f_2 frequency.

2.4. *Endocochlear potential*

Measurements of the endocochlear potential were performed as described previously (Kada et al., 2009). A silver–silver chloride reference electrode was placed under the skin of the dorsum. A micropipette electrode (10–40 MO) filled with 150 mM KCl was advanced through the bony aperture into the spiral ligament. The entry of the electrode tip into the endolymph was characterized by fast changes of the recorded potentials. The electrode was advanced until a stable potential was observed, at which point no alterations were dependent upon its depth. The signal was passed through an amplifier (Duo 773; World Precision Instruments, Sarasota, FL).

2.5. *Surgical procedure for topical application*

The EP4 antagonist ONO-AE3-208 was dissolved in 1 N NaOH and diluted with PBS to give a final concentration of 1 mg/ml and a final pH of 7.4. The EP4 agonist

ONO-AE1-329 was dissolved in DMSO and diluted with physiological saline to give a final concentration of 1 mg/ml containing 1% dimethyl sulfoxide (DMSO). Both drugs were applied topically under general anesthesia. The otic bulla of the left temporal bone was exposed via a retroauricular approach. A small hole was made in the otic bulla to access the round window membrane (RWM). A dry gelatin sponge was cut into 0.5–1 mm³ pieces and a piece of the sponge placed on the RWM following the immersion of substrates (2 µl). Control animals received topical application of PBS at pH 7.4 or physiological saline containing 1% DMSO.

2.6. *Noise exposure*

Animals under general anesthesia were placed in a ventilated sound-exposure chamber fitted with speakers driven by a noise generator and a power amplifier. A 1/2-inch condenser microphone and a fast Fourier transform analyzer (both from Sony, Tokyo, Japan) were used to monitor and calibrate sound levels at multiple locations within the chamber, in order to ensure uniformity of the stimulus. The stimulus intensity varied by a maximum of 3 dB SPL across measured sites within the exposure chamber.

2.7. *Histology*

After the functional analyses, the cochleae were subjected to histological analysis as whole mounts or 10- μ m-thick frozen sections. The whole-mount samples were used for the histological evaluation of hair cells. The frozen sections were used for the evaluation of gross anatomy by hematoxylin and eosin (H&E) staining, for immunohistochemistry for EP4 or for quantitative assessments of spiral ganglion neurons.

The whole-mount specimens were separated into three regions with distances from the apex of 20–40% (corresponding to 8–16 kHz regions; apical portion), 40–70% (corresponding to 16–32 kHz regions; mid-basal portion), and 70–90% (corresponding to 32–64 kHz regions; basal portion) (Viberg and Canlon, 2004). After permeabilization with 0.2% Triton X in PBS for 30 min at room temperature, the specimens were incubated with anti-myosin VIIa rabbit polyclonal antibody (1:500) for 12 h at 4 °C, followed by incubation with Alexa-568 conjugated anti-rabbit goat IgG (1:500) secondary antibody. At the end of the staining procedures, the specimens were stained with fluorescein–phalloidin (1:400) and 4',6-diamino-2-phenyl-indole (DAPI) for 15 min at room temperature, and viewed with a confocal microscope (TCS SPE; Leica Microsystems, Wetzlar, Germany). The respective numbers of inner hair cells (IHCs) and outer hair cells (OHCs) were counted, and the ratio of missing IHCs and OHCs was calculated for each region of cochleae.

Two mid-modiolar sections (separated by a distance of 40–50 μm) from each cochlea were used for immunostaining for EP4 or for the histological analysis of spiral ganglion neurons, respectively. Immunohistochemistry for EP4 was performed with anti-EP4 receptor (1:200) primary antibody and Alexa 568-conjugated anti-rabbit goat IgG (1:500) secondary antibody. Immunohistochemistry for βIII -tubulin was performed to identify the spiral ganglion neurons in the Rosenthal's canal. Specimens were treated with rabbit anti- βIII tubulin (1:250) primary antibody and Alexa-488 conjugated anti-rabbit goat IgG (1:500) secondary antibody, followed by nuclear labeling with DAPI. The specimens were then observed using a fluorescence microscope (Olympus BX50, Tokyo, Japan). The numbers of spiral ganglion neurons and the area of the Rosenthal's canal were quantified, and the density of spiral ganglion neurons was calculated as described previously (Kada et al., 2009).

2.8. *Statistical analysis*

Data are expressed as the mean \pm SEM for the indicated number of observations. The unpaired Student's *t*-test (two-tailed) was used, as appropriate, for comparisons between two groups. Two-way factorial analysis of variance (ANOVA) was used for comparisons of ABR-threshold shifts after noise exposure, and the Tukey-Kramer test

was performed for pair-wise comparisons. $P < 0.05$ was considered statistically significant.

3. Results

3.1. *Hearing loss in EP4-deficient mice*

ABR recordings for screening the auditory function of EP4-deficient mice demonstrated significant but slight hearing loss at frequencies of 10, 20, and 40 kHz. EP4-deficient mice (n=16) exhibited significant elevation of ABR thresholds in comparison with wild-type mice (n=16) at 10 kHz ($P < 0.0001$), 20 kHz ($P < 0.0001$), and 40 kHz ($P < 0.0001$) (Fig. 1A). To examine the mechanisms underlying the hearing impairment in EP4-deficient mice, we measured DPOAEs, which reflect the OHCs in the cochlea (Parham et al., 1999), and the endocochlear potential, which is the positive voltage in the endolymphatic space of the cochlea that is mainly generated by the stria vascularis (Tasaki and Spyropoulos, 1959).

In DPOAE measurements, EP4-deficient animals demonstrated significant loss in the DP/NF levels at f2 frequencies of 14,672 ($P < 0.0001$) and 15,984 Hz ($P = 0.0255$) compared with wild-type animals (n=16 for each; Fig. 1B). By contrast, the mean endocochlear potential of the EP4-deficient mice (107.3 ± 6.5 mV) did not differ significantly from that of wild-type mice (104.8 ± 5.1 mV; n=4 for each; Fig. 1C). These functional assessments suggested that the EP4-deficient mice had slight hearing loss due to OHC dysfunction.

To investigate the morphological phenotypes of the EP4-deficient mice, cochlear specimens were examined using either cryostat sections or surface preparations. The expression of EP4 was found in in the stria vascularis, spiral ganglion neurons, supporting cells, IHCs and OHCs in wild-type mice (Fig. 1D) similarly to our previous observation (Hori et al., 2009), while no expression of EP4 was identified in EP4-deficient mice (Fig. 1D). H&E staining of cross sections revealed that the gross anatomy of EP4-deficient mice cochleae was essentially normal (Fig. 1E). No apparent degeneration was seen in the organ of Corti, spiral ganglion, or cochlear lateral wall including the stria vascularis at the light-microscopic level. Immunostaining for myosin VIIa and f-actin labeling with phalloidin revealed the surface morphology of the organ of Corti. Loss of OHCs was observed in the basal, mid-basal, and apical portions of EP4-deficient cochleae (Fig. 1F). OHC loss was confirmed by the nuclear staining with DAPI. However, there was no statistically significant difference in OHC loss between EP4-deficient and wild-type mice (Fig. 1G). This finding suggested an involvement of other mechanisms besides OHC degeneration for hearing loss in EP4-deficient mice. We then quantitatively analyzed the density of spiral ganglion neurons, of which loss causes ABR threshold shifts, using immunostaining for β III tubulin in cross sections, which showed no significant loss of spiral ganglion neurons (Fig. 1H). We therefore

considered that EP4 deficiency affected auditory systems including OHC function, which might result in modest hearing loss under physiological conditions.

3.2. *Vulnerability to noise trauma in EP4-deficient mice*

Mouse models of noise-induced hearing loss have previously been used to test the roles of various molecules in pathophysiological conditions of the auditory system (Peppi et al., 2011; Polesskaya et al., 2010; 2 Tan et al., 2010). Noise-induced damage sometimes clarified roles of molecules in auditory function. We therefore examined the effects of EP4 deficiency on noise-induced hearing loss, in order to clarify its role in auditory function. EP4-deficient and wild-type mice (n=12 for each) were exposed to an octave-band noise centered on 8 kHz at a 120-dB SPL for 2 h, and then subjected to ABR measurements 7 and 14 days later. Alterations in the ABR-threshold shifts of EP4-deficient and wild-type mice are shown in Fig. 2A. The overall effects of EP4 deficiency on the ABR-threshold shifts were statistically significant at 10 kHz (df=1, $F=5.287$ and $P=0.0247$), 20 kHz (df=1, $F=10.720$ and $P=0.0017$), and 40 kHz (df=1, $F=17.323$ and $P<0.0001$) with two-way factorial ANOVA. Pair-wise comparisons with Tukey-Kramer test revealed significantly higher elevations of ABR thresholds in EP4-deficient than in wild-type mice on day 14 at 20 kHz, and on days 7 and 14 at 40

kHz. DPOAE assessments demonstrated significant decreases of DP/NF levels at 11,297 Hz ($P=0.0005$), 13,453 Hz ($P=0.0068$), 14,672 Hz ($P=0.0371$), and 15,984 Hz ($P=0.0006$) in EP4-deficient mice compared with wild-type mice (Fig. 2B). These findings demonstrated that noise exposure revealed auditory system differences between EP4-deficient and wild-type mice, in particular high frequency regions. By contrast, there was no significant difference in the endocochlear potential between EP4-deficient (103.3 ± 2.5 mV) and wild-type (97.3 ± 3.5 mV) mice (Fig. 2C).

Morphological analyses confirmed that EP4 deficiency enhanced the degeneration of OHCs in response to noise. Cochlear specimens were obtained 14 days after noise exposure. Noise-induced damage to the organ of Corti was assessed by immunostaining for myosin VIIa and f-actin labeling with phalloidin in whole-mount preparations. Minor loss of OHCs was observed in the basal, mid-basal, and apical portions of wild-type cochleae, whereas the EP4-deficient mice exhibited extensive loss of OHCs (Fig. 2D). A quantitative analysis demonstrated significant decreases of OHCs in the apical ($P=0.0058$), mid-basal ($P=0.0330$), and basal ($P=0.0400$) portions of the cochleae of EP4-deficient mice in comparison with those of wild-type mice (Fig. 2E). No significant difference in inner hair cells (IHCs) was found between EP4-deficient and wild-type mice (Fig. 2E). Immunostaining for β III tubulin in cross sections revealed

no degeneration of spiral ganglion neurons in either EP4-deficient or wild-type mice (Fig. 2F), and quantitative assessments revealed no significant difference in the density of spiral ganglion neurons between EP4-deficient and wild-type mice ($n=4$ for each; Fig. 2G). Consequently, morphological differences between EP4-deficient and wild-type mice also appeared to be apparent following noise exposure, which suggests that the OHC is included in targets of cochlear damage due to EP4 deficiency. These findings demonstrate that EP4-deficient mice are vulnerable to noise-induced damage in comparison with wild-type animals, indicating the importance of EP4 for cochlear protection, particularly in OHCs, against noise trauma.

3.3. *Inhibition of EP4 signaling enhanced noise-induced cochlear damage*

The pharmacological inhibition of EP4 signaling was investigated in order to confirm the effects of EP4 deficiency on noise-induced hearing loss. The EP4 antagonist ONO-AE3-208 (Kabashima et al., 2002; Sugimoto and Narumiya, 2007) was topically applied to the RWM in the middle-ear cavity of 8-week-old C57BL/6 mice. Immediately after the topical application, animals were exposed to an octave-band noise centered on 8 kHz at 120 dB SPL for 1 h. Both control and EP4 antagonist-treated animals ($n=5$ for each) showed similar elevations of ABR thresholds on day 1 after

noise exposure (Fig. 3A), indicating that both groups experienced similar levels of initial damage. However, the subsequent recovery process differed notably between the two groups (Fig. 3A). In control animals, the ABR-threshold shifts showed a trend towards recovery at all frequencies, whereas no recovery was observed in EP4 antagonist-treated animals (Fig. 3A). Statistical analyses revealed significant differences in the ABR-threshold shifts between the two groups at each frequency. The overall effects of EP4 antagonist application were significant at 10 kHz (df=1, $F=10.195$ and $P=0.0032$), 20 kHz (df=1, $F=10.662$ and $P=0.0026$), and 40 kHz (df=1, $F=15.703$ and $P=0.0004$). Pair-wise comparisons revealed significant differences in the ABR-threshold shifts on day 7 at 10 and 20 kHz, and on days 7 and 14 at 40 kHz. These findings demonstrate that pharmacological inhibition of EP4 had a similar effect to genetic deletion in enhancing noise-induced hearing loss.

Morphological assessment demonstrated the enhancement of noise-induced damage to OHCs caused by pharmacological inhibition of EP4 signaling. Immunostaining for myosin VIIa and f-actin labeling with phalloidin in whole-mount preparations revealed an enhancement of OHC loss in the basal, mid-basal, and apical portions of cochleae by the EP4 antagonist (Fig. 3B). A quantitative analysis demonstrated significantly greater OHC loss in the basal ($P=0.0473$), mid-basal

($P=0.0089$), and apical ($P=0.0282$) portions of cochleae treated with the EP4 antagonist (Fig. 3C). By contrast, no significant difference in IHC loss was found between the two groups (Fig. 3C). These findings revealed that pharmacological inhibition of EP4 had a similar effect to genetic deletion in enhancing OHC degeneration due to noise exposure. EP4 signaling might therefore play a role in protecting OHCs against noise-induced damage.

3.4. *EP4 agonist attenuated noise-induced cochlear damage*

Both genetic deletion and pharmacological inhibition of EP4 enhanced noise-induced damage to cochleae. These findings suggested that the activation of EP4 signaling could attenuate noise-induced hearing loss. We tested the effects of the EP4 agonist ONO-AE1-329 (Sugimoto and Narumiya, 2007; Suzawa et al., 2000) on noise-induced damage in 8-week-old C57BL/6 mice. Immediately after drug application, the animals were exposed to an octave-band noise centered on 8 kHz at 120 dB SPL for 2 h. Control animals ($n=5$) showed ABR-threshold shift of approximately 80 dB on day 7 and no recovery on day 14. The animals treated with the EP4 agonist ($n=5$) exhibited comparatively small ABR-threshold shifts on day 7, and showed a trend for decreasing shifts until day 14 (Fig. 4A). Statistical analyses revealed that local application of the

EP4 agonist had significant effects on ABR-threshold shifts at 10 kHz (df=1, $F=25.000$ and $P<0.0001$), 20 kHz (df=1, $F=9.164$ and $P=0.0058$), and 40 kHz (df=1, $F=36.152$ and $P<0.0001$). Pair-wise comparisons showed significant differences between the two groups in ABR-threshold shifts on days 7 and 14 at 10 kHz, on day 14 at 20 kHz, and on days 7 and 14 at 40 kHz. These findings demonstrate that the topical application of an EP4 agonist attenuated noise-induced hearing loss.

Morphologically, severe degeneration of OHCs was observed from the apex to the base of cochleae in control animals (Fig. 4B). Approximately 70% of the OHCs in the mid-basal and basal portions of cochleae were destroyed by the noise exposure in control animals (Fig. 4C). In contrast to control animals, OHCs in specimens treated with the EP4 agonist were well maintained (Fig. 4B). Quantitative assessments showed that the EP4 agonist had a significant protective effect on OHCs in the apical ($P=0.0209$), mid-basal ($P=0.0215$), and basal ($P=0.0455$) portions of cochleae (Fig. 4C). No significant difference in IHC loss was found between the two groups (Fig. 4C). These findings demonstrate that the pharmacological activation of EP4 signaling promoted the survival of OHCs exposed to noise trauma. These findings in pharmacological activation of EP4 support our hypothesis that EP4 signaling plays a crucial role in the maintenance of auditory function.

4. Discussion

The present study demonstrates that genetic deletion of EP4 causes slight elevation of ABR thresholds and loss of DPOAEs, although the endocochlear potential is maintained in normal levels. Genetic deletion of EP4 accelerated ABR threshold shifts and loss of DPOAEs, but not of endocochlear potentials, following noise exposure. These findings in EP4-deficient mice indicate that EP4 may be involved in the maintenance of auditory function, in particular OHC function. In addition, pharmacological inhibition of EP4 signaling enhanced noise-induced hearing loss, and its pharmacological activation attenuated noise-induced hearing loss. Findings in pharmacological inhibition or activation support the findings in genetic deletion of EP4.

Experimental animals were exposed to an octave-band noise centered on 8 kHz at a 120-dB SPL, which causes cochlear damage not only in low frequency regions, but also middle and high frequency regions (Ou et al., 2000), for 1 or 2 h according to the experimental design. In experiments of genetic deletion or pharmacological inhibition of EP4, we intended to induce mild hearing loss in control animals, which is suitable for assessments of acceleration of noise-induced damage by toxic treatments. In genetic deletion of EP4, 2-h noise exposure was used, while 1-h exposure was used in pharmacological inhibition of EP4, because of the difference in back ground strains

between two experiments. In genetic deletion of EP4, a mixed 129/Ola and C57BL/6 genetic background was used, while in pharmacological inhibition of EP4, we used C57BL/6 mice, which were reported to be more vulnerable to noise trauma than sub-strains of mouse strain 129 (Turner et al., 2005; Yoshida et al., 2000). We therefore used a shorter noise exposure period in pharmacological inhibition of EP4 than that in experiments using EP4-deficient mice. In experiments for pharmacological activation of EP4 signaling, we intended to generate profound hearing loss in control animals, which is suitable for assessments of protective effects. C57BL/6 mice were, therefore, exposed to an octave-band noise centered on 8 kHz at 120 dB SPL for 2 h, which was longer than that in pharmacological inhibition experiments. As we expected, profound hearing loss occurred in control animals, and significant protection by pharmacological activation of EP4 was observed.

The present study reveals that EP4 signaling plays a crucial role in the protection of OHCs against noise trauma. Extending the findings of our previous study that EP4 is expressed in the OHCs of the mouse cochlea (Hori et al., 2009), EP4 signaling could therefore act directly on OHCs. The activation of EP4 induces cyclic AMP production in OHCs, which might help to rescue them from energy depletion caused by overstimulation in response to excessive noise exposure. EP4 signaling has been

reported to activate anti-apoptotic pathways associated with protein kinase A (Hoshino et al., 2003), phosphatidylinositol 3-kinase-mediated AKT phosphorylation (Liou et al., 2007), BCL-2 antagonist of cell death (BAD) (Chun et al., 2007) and survivin (Baratelli et al., 2005). Such mechanisms could be involved in OHC protection by EP4 signaling in mouse models of noise-induced hearing loss.

The expression of EP4 was also identified in a variety of cell types in the mouse cochlea (Hori et al., 2009). Therefore, indirect effects of EP4 signaling could contribute to the promotion of the survival of OHCs. Previously, pharmacological activation of EP4 has been reported to induce generation of vascular endothelial growth factor in spiral ganglion neurons (Hori et al., 2010), which may contribute to the survival of OHCs against noise-induced damage (Picciotti et al., 2006; Selivanova et al., 2007).

There are some discrepancies between the functional and morphological findings in the present study. In EP4-deficient mice under a physiological condition, slight ABR threshold shifts were found, whereas morphological analyses demonstrated essentially normal morphology of cochleae except for limited loss of OHCs. C57/BL6 mice treated with an EP4 antagonist exhibited slight loss of OHCs, despite of remarkable ABR threshold shifts following noise exposure. These findings suggest that other targets of EP4 signaling in the cochlea besides OHCs may play a role in hearing loss due to EP4

deficiency. EP4 expression was also found in IHCs and spiral ganglion neurons, which are also involved in mechanisms for noise-induced hearing loss (Pujol and Puel, 1999). Hence, the IHC and spiral ganglion neuron could contribute to hearing loss due to genetic deletion or pharmacological inhibition of EP4. However, no significant loss of IHCs or spiral ganglion neurons was found in EP4-deficient mice under a physiological condition or after noise trauma in the present study. Therefore, degeneration at the substructural level might occur in IHCs and spiral ganglion neurons, in particular in afferent dendrites attached to the base of the IHC, which is known as a target of noise trauma (Pujol and Puel, 1999). Future studies using electron microscopy are required to elucidate detailed mechanisms underlying hearing loss due to lack of EP4 signaling.

In conclusion, the present findings demonstrate an involvement of EP4 signaling in the maintenance of the auditory system, and of OHCs in particular. The roles of other PGE receptors, including EP1–3, in the cochlea should also be determined, in order to understand the roles of PGE₂ signaling in the auditory system. This could help to identify new targets for cochlear disease therapeutics.

Acknowledgments

The authors thank Ono Pharmaceutical for supplying the EP4 agonist and antagonist.

This work was supported in partly by Grants for Research on Sensory and Communicative Disorders from the Japanese Ministry of Health, Labor, and Welfare, and a collaborative research grant to Kyoto University from Ono Pharmaceutical.

References

- Ahmad A.S., Ahmad M., de Brum-Fernandes A.J., Doré S., 2005. Prostaglandin EP4 receptor agonist protects against acute neurotoxicity. *Brain Res.* 1066, 71-77.
- Andreasson K., 2010. Emerging roles of PGE2 receptors in models of neurological disease. *Prostaglandins Other Lipid Mediat.* 91, 104-112.
- Baratelli F., Krysan K., Heuzé-Vourc'h N., Zhu L., Escudro B., Sharma S., Reckamp K., Dohadwala M., Dubinett SM., 2005. PGE2 confers survivin-dependent apoptosis resistance in human monocyte-derived dendritic cells. *J. Leukoc. Biol.* 78, 555-564.
- Chun K.S., Akunda J.K., Langenbach R., 2007. Cyclooxygenase-2 inhibits UVB-induced apoptosis in mouse skin by activating the prostaglandin E2 receptors, EP2 and EP4. *Cancer Res.* 67, 2015-2021.
- Esaki Y., Li Y., Sakata D., Yao C., Segi-Nishida E., Matsuoka T., Fukuda K., Narumiya S., 2010. Dual roles of PGE2-EP4 signaling in mouse experimental autoimmune encephalomyelitis. *Proc. Natl. Acad. Sci. U. S. A.* 107, 12233-12238.
- Funk C.D., 2001. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science.* 294, 1871-1875.
- Harris S.G., Padilla J., Koumas L., Ray D., Phipps R.P., 2002. Prostaglandins as modulators of immunity. *Trends Immunol.* 23, 144-150.

- Hori R., Nakagawa T., Sugimoto Y., Sakamoto T., Yamamoto N., Hamaguchi K., Ito J., 2009. Prostaglandin E receptor subtype EP4 agonist protects cochleae against noise-induced trauma. *Neuroscience*. 160, 813-819.
- Hori R., Nakagawa T., Yamamoto N., Hamaguchi K., Ito J., 2010. Role of prostaglandin E receptor subtypes EP2 and EP4 in autocrine and paracrine functions of vascular endothelial growth factor in the inner ear. *BMC Neurosci*. 11:35.
- Hoshino T., Tsutsumi S., Tomisato W., Hwang H.J., Tsuchiya T., Mizushima T., 2003. Prostaglandin E2 protects gastric mucosal cells from apoptosis via EP2 and EP4 receptor activation. *J. Biol. Chem.* 278, 12752-12758.
- Kabashima K., Saji T., Murata T., Nagamachi M., Matsuoka T., Segi E., Tsuboi K., Sugimoto Y., Kobayashi T., Miyachi Y., Ichikawa A., Narumiya S., 2002. The prostaglandin receptor EP4 suppresses colitis, mucosal damage and CD4 cell activation in the gut. *J. Clin. Invest.* 109, 883-893.
- Kada S., Nakagawa T., Ito J., 2009. A mouse model for degeneration of the spiral ligament. *J. Assoc. Res. Otolaryngol.* 10, 161-172.
- Kawata R., Urade Y., Tachibana M., Mizukoshi O., 1988. Prostaglandin synthesis by the cochlea. *Prostaglandins*. 35, 173-184.
- Legler D.F., Bruckner M., Uetz-von Allmen E., Krause P., 2010. Prostaglandin E2 at

- new glance: novel insights in functional diversity offer therapeutic chances. *Int. J. Biochem. Cell Biol.* 42, 198-201.
- Li J., Liang X., Wang Q., Breyer R.M., McCullough L., Andreasson K., 2008. Misoprostol, an anti-ulcer agent and PGE2 receptor agonist, protects against cerebral ischemia. *Neurosci. Lett.* 438, 210-215.
- Liou J.Y., Ellent D.P., Lee S., Goldsby J., Ko B.S., Matijevic N., Huang J.C., Wu K.K., 2007. Cyclooxygenase-2-derived prostaglandin e2 protects mouse embryonic stem cells from apoptosis. *Stem Cells.* 25, 1096-1103.
- Nakagawa T., 2011. Roles of prostaglandin E2 in the cochlea. *Hear. Res.* 276, 27-33.
- Nakashima T., Naganawa S., Sone M., Tominaga M., Hayashi H., Yamamoto H., Liu X., Nuttall A.L., 2003. Disorders of cochlear blood flow. *Brain Res. Rev.* 43, 17-28.
- Narumiya S., 2007. Physiology and pathophysiology of prostanoid receptors. *Proc. Jpn. Acad. Ser. B.* 83, 296-319
- Nguyen M., Camenisch T., Snouwaert J.N., Hicks E., Coffman T.M., Anderson P.A., Malouf N.N., Koller B.H., 1997. The prostaglandin receptor EP4 triggers remodelling of the cardiovascular system at birth. *Nature.* 390, 78-81.
- Ou H.C., Bohné B.A., Harding G.W., 2000. Noise damage in the C57BL/CBA mouse cochlea. *Hear. Res.* 145, 111-122.

- Parham K., Sun X.M., Kim D.O., 1999. Distortion product otoacoustic emissions in the CBA/J mouse model of presbycusis. *Hear. Res.* 134, 29-38.
- Peppi M., Kujawa S.G., Sewell W.F., 2011. A corticosteroid-responsive transcription factor, promyelocytic leukemia zinc finger protein, mediates protection of the cochlea from acoustic trauma. *J. Neurosci.* 31, 735-741.
- Picciotti P.M., Fetoni A.R., Paludetti G., Wolf F.I., Torsello A., Troiani D., Ferraresi A., Pola R., Sergi B., 2006. Vascular endothelial growth factor (VEGF) expression in noise-induced hearing loss. *Hear. Res.* 214, 76-83.
- Polesskaya O., Cunningham L.L., Francis S.P., Luebke A.E., Zhu X., Collins D., Vasilyeva O.N., Sahler J., Desmet E.A., Gelbard H.A., Maggirwar S.B., Walton J.P., Frisina R.D. Jr., Dewhurst S., 2010. Ablation of mixed lineage kinase 3 (Mlk3) does not inhibit ototoxicity induced by acoustic trauma or aminoglycoside exposure. *Hear. Res.* 270, 21-27.
- Pujol R., Puel J.L., 1999. Excitotoxicity, synaptic repair, and functional recovery in the mammalian cochlea: a review of recent findings. *Ann. N. Y. Acad. Sci.* 884, 249-254.
- Rhee C.K., Park Y.S., Jung T.T., Park C.I., 1999. Effects of leukotrienes and prostaglandins on cochlear blood flow in the chinchilla. *Eur. Arch. Otorhinolaryngol.*

256, 479-483.

- Segi E., Sugimoto Y., Yamasaki A., Aze Y., Oida H., Nishimura T., Murata T., Matsuoka T., Ushikubi F., Hirose M., Tanaka T., Yoshida N., Narumiya S., Ichikawa A., 1998. Patent ductus arteriosus and neonatal death in prostaglandin receptor EP4-deficient mice. *Biochem. Biophys. Res. Commun.* 246, 7-12.
- Selivanova O., Heinrich U.R., Brieger J., Feltens R., Mann W., 2007. Fast alterations of vascular endothelial growth factor (VEGF) expression and that of its receptors (Flt-1, Flk-1 and Neuropilin) in the cochlea of guinea pigs after moderate noise exposure. *Eur. Arch. Otorhinolaryngol.* 264, 121-128.
- Shi J., Johansson J., Woodling N.S., Wang Q., Montine T.J., Andreasson K., 2010. The prostaglandin E2 E-prostanoid 4 receptor exerts anti-inflammatory effects in brain innate immunity. *J. Immunol.* 184, 7207-7218.
- Simmons D.L., Botting R.M., Hla T., 2004. Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. *Pharmacol. Rev.* 56, 387-437.
- Smith W.L., DeWitt D.L., Garavito R.M., 2000. Cyclooxygenases: structural, cellular, and molecular biology. *Annu. Rev. Biochem.* 69, 145-182.
- Stjernschantz J., Wentzel P., Rask-Andersen H., 2004. Localization of prostanoid receptors and cyclo-oxygenase enzymes in guinea pig and human cochlea. *Hear. Res.*

197, 65-73.

Sugimoto Y., Narumiya S., 2007. Prostaglandin E receptors. *J. Biol. Chem.* 282, 11613-11617.

Suzawa T., Miyaura C., Inada M., Maruyama T., Sugimoto Y., Ushikubi F., Ichikawa A., Narumiya S., Suda T., 2000. The role of prostaglandin E receptor subtypes (EP1, EP2, EP3, and EP4) in bone resorption: an analysis using specific agonists for the respective EPs. *Endocrinology*. 141, 1554-1559.

Tan J., Clarke M., Barrett G., Millard R., 2010. The p75 neurotrophin receptor protects primary auditory neurons against acoustic trauma in mice. *Hear. Res.* 268, 46-59.

Tasaki I, Spyropoulos C.S., 1959. Stria vascularis as source of endocochlear potential. *J. Neurophysiol.* 1959. 22, 149-155.

Tominaga M., Yamamoto H., Sone M., Teranishi M.A., Nakashima T., 2006. Response of cochlear blood flow to prostaglandin E1 applied topically to the round window. *Acta Otolaryngol.* 126, 232-236.

Turner J.G., Parrish J.L., Hughes L.F., Toth L.A., Caspary D.M., 2005. Hearing in laboratory animals: strain differences and nonauditory effects of noise. *Comp. Med.* 55, 12-23.

Umemura K., Takiguchi Y., Nakashima M., Nozue M., 1990. Effect of arachidonic acid

on the inner ear blood flow measured with a laser Doppler flowmeter. *Ann. Otol.*

Rhinol. Laryngol. 99, 491-495.

Umemura T., Harada N., Kitamura T., Ishikura H., Okajima K., 2010. Limaprost reduces motor disturbances by increasing the production of insulin-like growth factor I in rats subjected to spinal cord injury. *Transl. Res.* 156, 292-301.

Viberg A., Canlon B., 2004. The guide to plotting a cochleogram. *Hear. Res.* 2004 197, 1-10.

Yoshida N., Hequembourg S.J., Atencio C.A., Rosowski J.J., Liberman M.C., 2000. Acoustic injury in mice: 129/SvEv is exceptionally resistant to noise-induced hearing loss. *Hear. Res.* 141, 97-106.

Figure Captions

Fig. 1. Auditory function and cochlear morphology of EP4-deficient mice. (A) The ABR thresholds in EP4-deficient mice differed significantly at each frequency in comparison with those in wild-type mice. Asterisk indicates a significant difference ($P<0.05$). (B) The DP/NF levels of EP4-deficient mice were significantly lower than those of wild-type mice at f2 frequencies of 15,984 and 14,672 Hz. Asterisk indicates a significant difference ($P<0.05$). (C) No statistical difference in the endocochlear potential was found between EP4-deficient and wild-type mice. (D) Immunostaining for EP4 (red) and nuclear staining with DAPI (blue) showed EP4 expression in an inner hair cell (I) and outer hair cells (O) of the organ of Corti, spiral ganglion neurons and stria vascularis in a wild-type cochlea, while no expression was found in an EP4-deficient cochlea. Scale bar, 50 μ m. (E) H&E staining of a cross section of an EP4-deficient cochlea revealed no abnormalities. OC, organ of Corti; RM, Reissner's membrane; SGN, spiral ganglion neuron; SV, stria vascularis. Scale bars, 200 μ m. (F) Immunostaining for myosin VIIa (red) and F-actin labeling with phalloidin (green) revealed loss of OHCs in EP4-deficient mice (asterisked). Scale bar, 50 μ m. (G) The percentages of OHC loss in each part of cochleae in EP4-deficient and wild-type mice

are shown. (H) No significant differences in the densities of spiral ganglion neuron (SGN) were found in the apical (Ap), mid-basal (mB) or basal (Bs) portions of cochleae between EP4-deficient and wild-type mice. In all graphs, the error bars represent the SEM.

Fig. 2. Vulnerability of EP4-deficient mice to noise trauma (an octave-band noise centered on 8 kHz at a 120-dB SPL for 2 h). (A) The time courses of the alterations in the ABR-threshold shifts of EP4-deficient and wild-type mice at frequencies of 10, 20, and 40 kHz are shown. The overall effects of EP4 deficiency on the ABR-threshold shifts were statistically significant at all frequencies. Asterisks indicate significant differences in ABR-threshold shifts in pair-wise comparisons. (B) Significant decreases in DP/NF levels in EP4-deficient mice were observed at 11,297, 13,453, 14,672 and 15,984 Hz. Asterisk indicates a significant difference ($P<0.05$). (C) The endocochlear potential in EP4-deficient mice was similar to that in wild-type mice after noise trauma. (D) Immunostaining for myosin VIIa (red) and F-actin labeling with phalloidin (green) revealed extensive loss of OHCs in each cochlear portion of EP4-deficient mice (asterisked). Scale bar, 20 μ m. (E) EP4-deficient mice showed significantly higher levels of OHC loss in the apical (Ap), mid-basal (mB), and basal (Bs) portions of

cochleae compared with wild-type mice. Asterisk indicates a significant difference ($P < 0.05$). (F) Immunostaining for β III-tubulin (green) and nuclear staining for DAPI (blue) revealed the spiral ganglion neurons of EP4-deficient mice to be normal. Scale bar, 50 μ m. (G) There was no significant difference in the density of spiral ganglion neurons (SGN) between EP4-deficient and wild-type mice. In all graphs, the error bars represent the SEM.

Fig. 3. Pharmacological inhibition of EP4 accelerated noise-induced damage in cochleae. The EP4 antagonist ONO-AE3-208 was topically applied to the RWM and control mice received a topical application of PBS at pH 7.4. (A) The time courses of the alterations in the ABR-threshold shifts of EP4 antagonist-treated and control mice at frequencies of 10, 20, and 40 kHz are shown. The overall effects of EP4 antagonist application were significant at all frequencies. Asterisks indicate significant differences in ABR-threshold shifts between EP4 antagonist-treated and control mice with pair-wise comparisons. (B) Immunostaining for myosin VIIa (red) and F-actin labeling with phalloidin (green) demonstrated extensive loss of OHCs in the basal, mid-basal, and apical portions of cochleae treated with the EP4 antagonist. Scale bar, 20 μ m. (C) A quantitative analysis demonstrated significant differences between the two groups in the

numbers of lost OHCs in the apical, mid-basal, and basal portions of cochleae. Asterisk indicates a significant difference ($P<0.05$). No significant difference in IHC numbers was found between the two groups. In all graphs, the error bars represent the SEM.

Fig. 4. The EP4 agonist ameliorated noise-induced damage to cochleae. The EP4 agonist ONO-AE1-329 was locally applied to the RWM and control animals received a local application of physiological saline containing 1% DMSO. (A) The time courses of the alterations in the ABR-threshold shifts of EP4 agonist-treated and control mice at frequencies of 10, 20, and 40 kHz are shown. The overall effects of local application of the EP4 agonist on ABR-threshold shifts were significant at all frequencies. Asterisks indicate significant differences in ABR-threshold shifts between EP4 agonist-treated and control mice with pair-wise comparisons. (B) Immunostaining for myosin VIIa (red) and F-actin labeling with phalloidin (green) showed severe loss of OHCs in control specimens, whereas the morphology of the organ of Corti was preserved in EP4 agonist-treated mice. Scale bar, 20 μ m. (C) A quantitative analysis demonstrated significant differences between the two groups in the numbers of lost OHCs in the apical, mid-basal, and basal portions of cochleae. Asterisk indicates a significant difference ($P<0.05$). No significant difference in IHC numbers was found between the

two groups. In all graphs, the error bars represent the SEM.

Highlights

> EP4-deficient mice exhibit modest hearing loss and are vulnerable to noise trauma. >
Pharmacological inhibition of EP4 has a similar effect to genetic deletion. >
Pharmacological activation of EP4 attenuates noise-induced cochlear damage. > EP4
signaling may be involved in the maintenance of auditory function

Figure1

[Click here to download high resolution image](#)

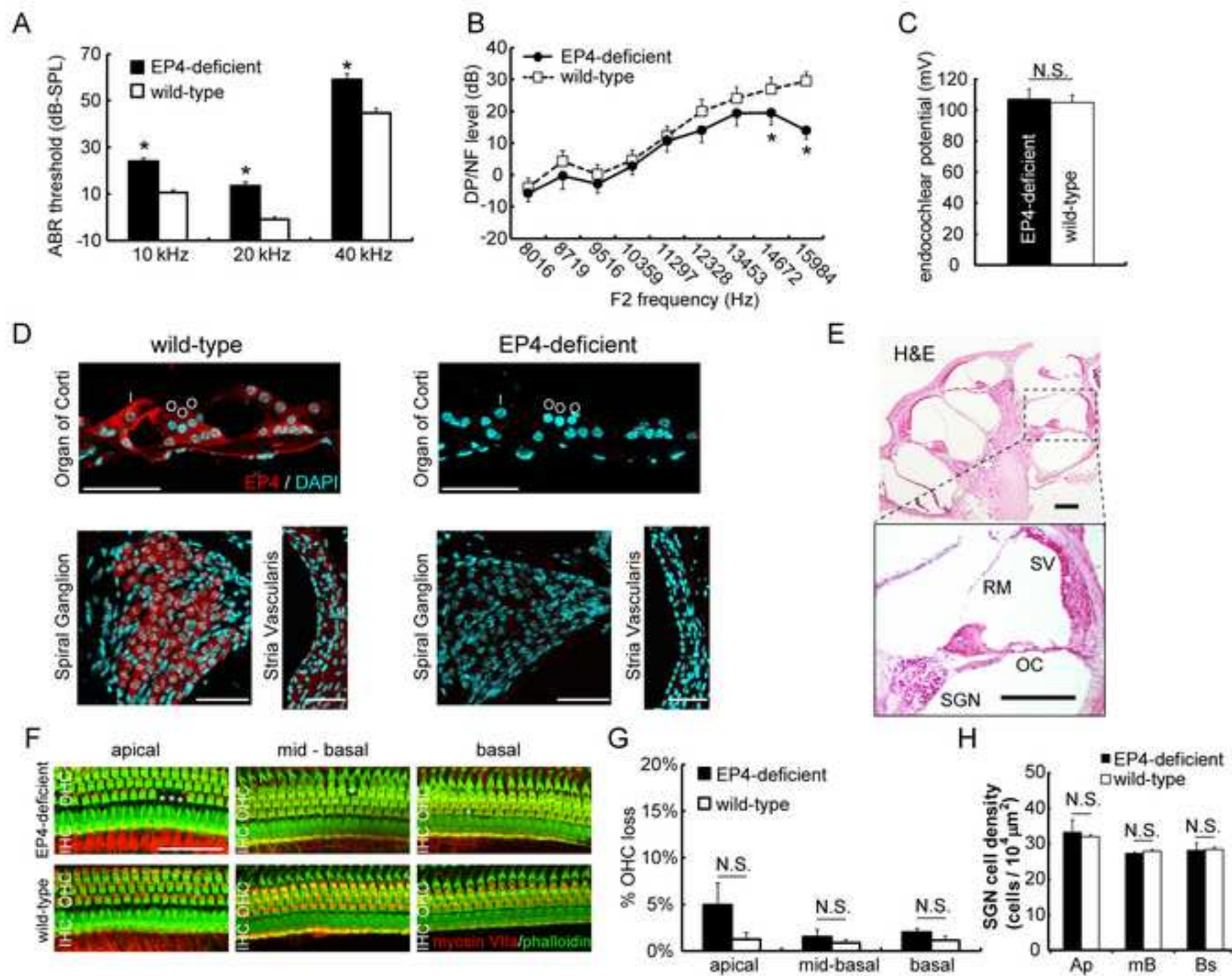


Figure 2

[Click here to download high resolution image](#)

